

AD-A268 489 INFORMATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE 1993		3. REPORT TYPE AND DATES COVERED Reprint																					
4. TITLE AND SUBTITLE (see title on reprint)				5. FUNDING NUMBERS PE: NWED QAXM WU: 4110																					
6. AUTHOR(S) Ramakrishnan et al.																									
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Armed Forces Radiobiology Research Institute 8901 Wisconsin Ave. Bethesda, MD 20889-5603				8. PERFORMING ORGANIZATION REPORT NUMBER SR93-17																					
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Defense Nuclear Agency 6801 Telegraph Road Alexandria, VA 22310-3398				10. SPONSORING/MONITORING AGENCY REPORT NUMBER																					
11. SUPPLEMENTARY NOTES																									
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.				12b. DISTRIBUTION CODE																					
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Membranes as sensitive targets in thymocyte apoptosis

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(Received 4 November 1992; revision received 8 February 1993; accepted 11 February 1993)

Abstract. The role of cellular membranes in thymocyte apoptosis has been examined. Trolox, a water soluble analogue of vitamin E and inhibitor of membrane damage, inhibits DNA fragmentation in thymocytes exposed to γ -radiation. Trolox is most effective in inhibiting DNA fragmentation when added to cells within 30 min post-irradiation. Exposure to trolox only during irradiation did not prevent DNA fragmentation, suggesting that it does not work by scavenging free radicals generated during radiation exposure. Incubation of the irradiated cell suspension with trolox for 2 h post-irradiation was sufficient to prevent DNA fragmentation measured at 24 h in irradiated cells. This suggests that trolox irreversibly inhibits a cellular lesion required for apoptosis. The induction of DNA fragmentation appears to be related to a concurrent, pronounced flow of Ca^{2+} into the cell. At 3 h post-irradiation the amount of Ca^{2+} in irradiated thymocytes was more than twice that of unirradiated thymocytes. Membrane damage has been shown to affect the transport of Ca^{2+} . Trolox treatment completely blocked the radiation-induced influx of Ca^{2+} into the thymocytes. These results suggest that membrane damage is a critical lesion that is involved in DNA fragmentation in thymocyte apoptosis.

1. Introduction

Thymocytes are among the most radiation sensitive cells in the body. At clinically relevant doses of radiation they die by a process termed interphase death or apoptosis (Maruyama and Feola 1987, Sellins and Cohen 1987). Apoptosis can also be induced by other physical agents, including hyperthermia (Sellins and Cohen 1991, Harmon *et al.* 1991), UV irradiation (Martin and Cotter 1991, Servoma and Rytoma 1990), and the photodynamic action of phthalocyanines (Agarwal *et al.* 1991). Agents such as cytotoxic T-cells (Martz and Howell 1989), antibodies against CD3 (Smith *et al.* 1989) and other molecules of the cell membrane (Trauth *et al.* 1989), or glucocorticoids (Wyllie 1980) induce apoptosis in target cells by receptor-mediated processes. Apoptosis is distinguishable from the more

common necrotic death, which is characterized by a generalized breakdown of cellular structure and function. Apoptotic death is, on the other hand, a physiological response to stimuli that has also been called cellular suicide or programmed cell death (Kerr *et al.* 1972).

Even though several morphological and biochemical changes have been described in apoptosis, it is not clear which biological systems or molecules participate in the process, or how their damage results in cell death. The most characteristic biochemical event during apoptosis is fragmentation of nuclear DNA into oligonucleosomal subunits that precedes cell death (Ramakrishnan and Catravas 1992a, Cohen and Duke 1984, Smith *et al.* 1989, Bellomo *et al.* 1992). The fragmentation of nuclear DNA during apoptosis appears to be due to activation of a Ca^{2+} -dependent nuclear endonuclease that is constitutively present in an inactive form in thymocyte nuclei (Nikonova *et al.* 1982, Cohen and Duke 1984, Kaminskas and Li 1989, Ramakrishnan and Catravas 1992a). The process by which this enzyme becomes activated is unknown, though there is an increase in intracellular Ca^{2+} concentration during apoptosis (McConkey *et al.* 1988, 1989a, 1990, Story *et al.* 1992). The integrity of the plasma membrane plays an important role in maintaining the Ca^{2+} homeostasis in the cell (Lucy 1972, Pascoe and Reed 1989).

An essential role for the lymphocyte plasma membrane in the development of apoptosis has been proposed (Konings 1981, Ashwell *et al.* 1986, Sungurov and Sharlaeva 1988). In irradiated thymocytes the onset of nuclear DNA fragmentation is preceded by changes in the structure and function of cellular membranes (Zherbin and Chukhlovina 1984, Yamada *et al.* 1969, Chandra and Stefani 1981, Sungurov and Sharlaeva 1988). The permeability of the plasma membrane and cell volume increases following γ -irradiation of lymphocytes, which may be mediated by an oxygen-dependent free-radical chain reaction (Ashwell *et al.* 1986). Lymphocyte survival following γ -irradiation is inversely dose-rate dependent, and may be due to radiation-induced damage to the plasma membrane of the cell (Kon-

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ings 1981). The expression of surface IgG on B cells is highly radiosensitive and involves activation of protein kinase C (Ojeda *et al.* 1991). Recently, it has been reported that protein kinase C is activated during apoptosis induced by γ -irradiation (Ojeda *et al.* 1992) and glucocorticoids (Ojeda *et al.* 1990). This activation of protein kinase C may be related to increases in diacylglycerol, one of the earliest signal-induced breakdown products of membrane-bound inositol phospholipid.

In this study we used trolox to investigate the involvement of membranes in DNA fragmentation in thymocytes exposed to γ -radiation. Trolox is a water-soluble analogue of vitamin E that penetrates biomembranes rapidly (Doba *et al.* 1985, Castle and Perkins 1986). It is a powerful inhibitor of membrane damage and protects mammalian cells from oxidative damage both *in vivo* (Casini *et al.* 1985, Mickle *et al.* 1989) and *in vitro* (Wu *et al.* 1990). Our results show that exposing thymocytes to trolox after irradiation blocks DNA fragmentation. The radiation-induced influx of extracellular Ca^{2+} is also inhibited in trolox-treated cells.

2. Materials and methods

2.1. Thymocyte isolation

CD2F1 male mice (6–7 weeks old) were killed with CO_2 and their thymuses removed and placed in ice-cold culture medium [RPMI 1640 medium supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, all from GIBCO/BRL (Grand Island, NY, USA) and 10% heat-inactivated foetal calf serum (HyClone Laboratories, Logan, UT, USA)]. Single-cell suspensions were prepared by pressing the organs through wire mesh screens followed by passage through a 25-gauge needle. The cells were washed once and resuspended in medium. Viable cells were determined by their ability to exclude trypan blue (Warters 1992).

2.2. Irradiation

Thymocytes suspended in medium ($2 \times 10^6/\text{ml}$) were exposed to 1.5–6.0 Gy ^{60}Co γ -radiation at a nominal dose rate of 1 Gy/min using the AFRRI cobalt facility.

2.3. Trolox treatment

Immediately after irradiation, cells were centrifuged at 200 *g* for 10 min, resuspended in fresh medium at 2×10^6 cells/ml, and incubated with trolox (Hoffman-LaRoche, Nutley, NJ, USA) at 37°C under an atmosphere of 5% CO_2 in air. It was necessary to prepare stock concentrations of trolox (100 mM) in 1 M NaHCO_3 because of its poor solubility in water above 1.8 mM (Wu *et al.* 1990). The pH of the stock was adjusted to 7.0 with 1 N HCl, and the stock was diluted to working concentrations with medium.

Dexamethazone was dissolved in a minimal volume of ethanol and diluted to the desired concentration with culture medium. Thymocytes were incubated with dexamethazone with or without trolox as described above. A similar volume of ethanol was added to controls.

2.4. DNA fragmentation assay

DNA fragmentation was assayed as previously described (Ramakrishnan and Catravas 1992a). At selected times cells were harvested by centrifugation at 200 *g* for 10 min. The cells were lysed with 0.2 ml ice-cold hypotonic 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100 (lysis buffer) and centrifuged at 13 000 *g* for 20 min to separate intact from fragmented DNA. The pellet was then sonicated for 10 s in 0.2 ml lysis buffer. DNA in the pellet and supernatant fractions was determined by an automated fluorometric method using Hoechst 33258 fluorochrome (Calbiochem-Behring, La Jolla, CA, USA) (Brunk *et al.* 1979, Cesarone *et al.* 1979), modified for our studies. The method utilized the Technicon Autoanalyzer II components (Technicon Instruments Corp., Tarrytown, NY, USA), including an autosampler fitted with a 40-place sample tray, a single-speed proportioning pump, and a fluoronephelometer. All tubes were flow-rated Tygon tubing (Fisher Scientific, Pittsburg, PA, USA). The sampler cam permitted the analysis of 40 samples/h with a 1 min running buffer wash between 30-s sample draws. The fluorescence signal was directed to a Hewlett-Packard 3390A integrator (Downer's Grove, IL, USA), which automatically identified and quantitated sample peaks.

The concentration of DNA was determined with computer software that compared the sample peak height value with a standard curve of peak heights of known concentrations of calf thymus DNA. The fluorometric autoanalysis of DNA is more sensitive

and reproducible than the diphenylamine method. Sample concentrations of 1–20 µg/ml were easily analysed with the system. Increased sensitivity can be obtained by increasing the volume of the sample draw and/or adjusting the sensitivity of the fluoronephelometer and integrator. Measurements were unaffected by the presence of cell homogenates or reagents in the sample.

The percentage of DNA fragmentation refers to the ratio of DNA in the 13 000 *g* supernatant to the total DNA in the pellet and 13 000 *g* supernatant.

2.5. DNA electrophoresis

Thymocytes were lysed in lysis buffer and incubated with proteinase K (50 µg/ml at 37°C for 30–45 min). The DNA was sequentially extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was precipitated overnight at –20°C in 100% ethanol. DNA was collected by centrifugation at 13 000 *g* for 20 min, air dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.8. Horizontal electrophoresis of DNA was performed for 2 h at 100 V in a 0.75% agarose gel with 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0 as running buffer (Ramakrishnan and Catravas 1992a).

2.6. Calcium measurements

Ca²⁺ uptake studies were performed by a method modified from McClain *et al.* (1984). Thymocytes (1 × 10⁷/ml) were incubated at 37°C for 30 min prior to radiation exposure in medium containing 10 µCi ⁴⁵Ca²⁺/ml (⁴⁵CaCl₂, 28.6 mCi/mg, Dupont/NEN, Wilmington, DE, USA). This preincubation time was sufficient to equilibrate Ca²⁺ stores in the thymocytes with the isotope (unpublished observations). After irradiation the cell suspension was returned to 37°C and incubated with gentle mixing. At selected times post-irradiation aliquots of the cell suspension (50 µl, 5 × 10⁵ cells) were removed and layered over 150 µl of a silicone oil mixture [Versilube F50, General Electric, Waterford, NY, USA with 8% (v/v) light mineral oil (Fisher Scientific)] in a 0.6 ml micro-centrifuge tube. The sample was centrifuged at 13 500 *g* in a microfuge for 45 s to pellet the cells through the oil, separating them from the radioactive medium. The aqueous and oil layers were carefully aspirated, and the cell pellet was resuspended in Hanks' Balanced Salt Solution (GIBCO/BRL) containing 1% Triton X-100. The samples were transferred to 7-ml scintillation vials. The precision of replicates was greatly improved by

also adding to each vial the pipet tip used to transfer the sample and the bottom 75–100 µl volume of the micro-centrifuge tube (cut with a Micro Tube Cutter, Thomas Scientific, Swedesboro, NJ, USA). Ecoscint A biodegradable scintillation solution (7 ml) (National Diagnostics, Manville, NJ, USA) was added to each scintillation vial, the vial contents vigorously shaken, and the radioactivity in the samples counted on a scintillation counter (LS5801, Beckman Instruments, Fullerton, CA, USA).

The amount of Ca²⁺ associated with the cells was calculated from the cpm in the cell pellet divided by the specific activity of ⁴⁵Ca²⁺ in the incubation medium. The specific activity was calculated by dividing the cpm in 10 µl of the radioactive cell suspension by the Ca²⁺ concentration in the incubation medium (0.42 mM). The quantity of Ca²⁺ associated with the cell pellet was normalized to that in 1 × 10⁶ cells.

3. Results

The effect of trolox on DNA fragmentation in thymocytes exposed to increasing doses of γ-radiation is shown in Figure 1a and b. There was no DNA fragmentation in irradiated thymocytes immediately after irradiation (6 Gy data; Figure 1a). DNA fragmentation began in irradiated thymocytes 2–3 h following irradiation and increased with time. This fragmentation was completely blocked by trolox following the post-irradiation incubation. There was a 10–20% background DNA fragmentation in unirradiated thymocytes not treated with trolox following an 8-h incubation at 37°C (Figure 1a and b). Incubation of unirradiated thymocytes with trolox reduced even the background fragmentation to negligible levels. Exposure of the cells to trolox did not alter significantly the viability of the cells. After an 8-h incubation with 10 mM trolox at 37°C under an atmosphere of 5% CO₂, 85 ± 3% (mean ± sem, *n* = 3) of the thymocytes retained the ability to exclude trypan blue. This compared with a value of 90 ± 3% (*n* = 3) for cells incubated under the same conditions without trolox.

Electrophoretic analysis of DNA isolated from thymocytes irradiated with 3 Gy showed a typical 'ladder' pattern of DNA fragments that were multiples of 200 base pairs (lane 4, Figure 2). The DNA isolated from irradiated thymocytes following trolox treatment showed no fragmentation (lane 5). The DNA of unirradiated thymocytes contained a small degree of fragmentation (lane 2), and there were no fragments in the DNA isolated from unirradiated trolox-treated thymocytes (lane 3). The results indi-

cate that the fragmentation of nuclear DNA into oligonucleosomal subunits, the most important biochemical event in apoptosis, can be inhibited by trolox.

Figure 3 shows the effect of different concentrations of trolox on DNA fragmentation in thymocytes

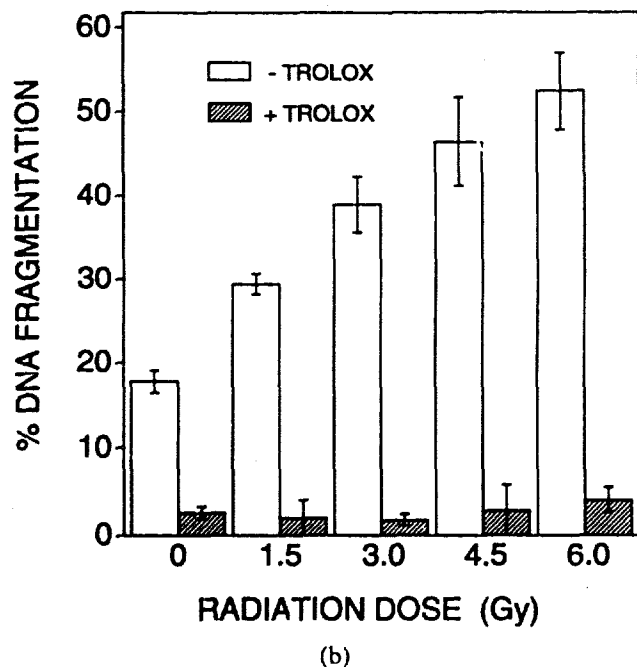
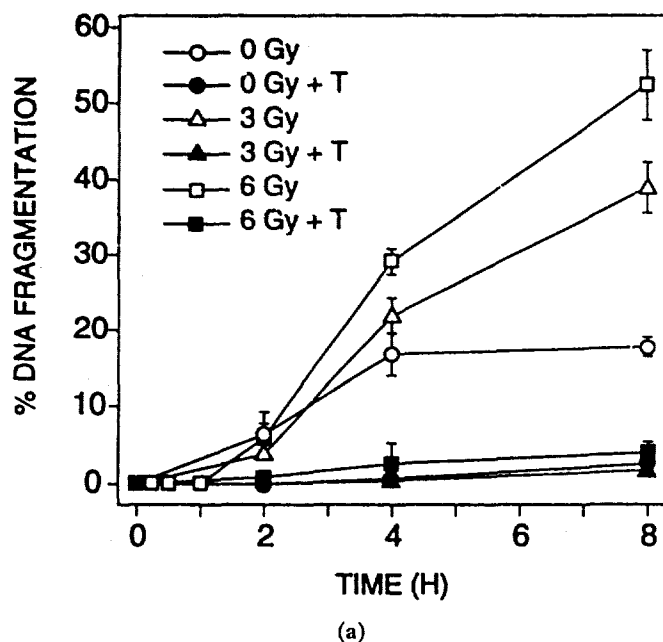


Figure 1. DNA fragmentation in irradiated thymocytes and inhibition by trolox. (a), at different post-irradiation times; (b), at 8 h after increasing doses of γ -irradiation. Thymocytes (2×10^6 cells/ml) were irradiated at a dose rate of 1 Gy/min. After irradiation cells were incubated in fresh medium with or without 10 mM trolox at 37°C under an atmosphere of 5% CO_2 in air. At indicated times DNA fragmentation was determined as described in §2. The results are mean \pm SE from three independent experiments ($n=6$). T, trolox.

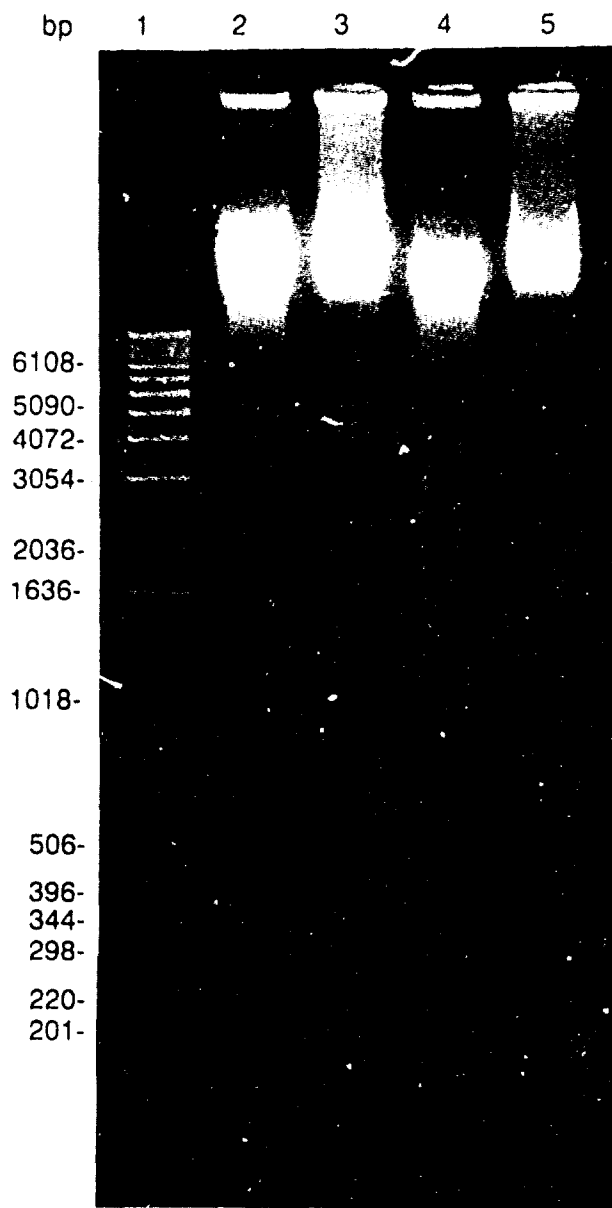


Figure 2. Trolox inhibits DNA fragmentation. DNA was isolated from unirradiated (0 Gy) and irradiated (3 Gy) thymocytes after 6-h incubation with or without 10 mM trolox and analysed by agarose gel electrophoresis. Lane 1, standard 1 kb DNA ladder; lane 2, 0 Gy; lane 3, 0 Gy + trolox; lane 4, 3 Gy; and lane 5, 3 Gy + trolox.

exposed to 6 Gy. The inhibition of fragmentation by trolox depended on its concentration in the incubation medium. Because DNA fragmentation was completely blocked by 10 mM trolox we used this concentration in all subsequent studies.

In the studies above, trolox was present in the medium during the entire post-irradiation incubation period. It was possible, however, that the effectiveness of trolox required it to be present only at a certain stage during the sequence of steps leading to DNA fragmentation. We therefore performed a series of experiments to determine how long trolox must

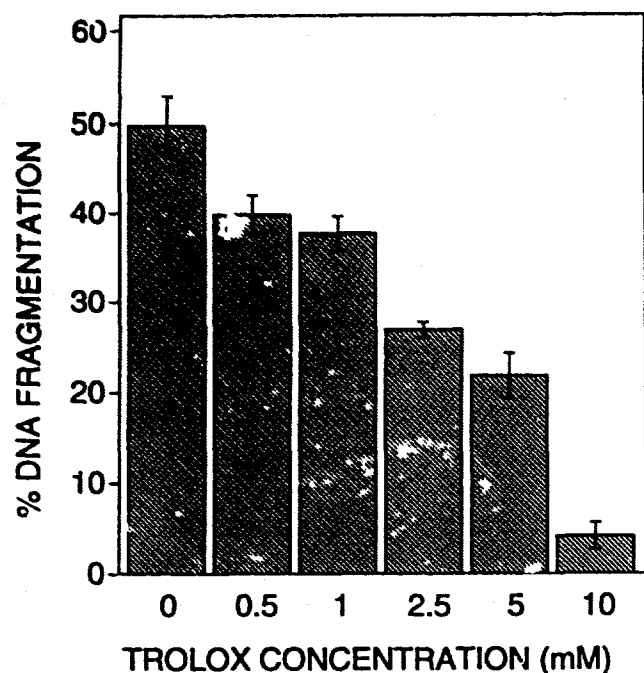


Figure 3. Inhibition of DNA fragmentation depends on concentration of trolox. Irradiated thymocytes (6 Gy) were incubated in fresh medium with different concentrations of trolox as described in Figure 1. DNA fragmentation was determined 8 h after irradiation. The results are mean \pm SE from three independent experiments ($n=6$).

be present after irradiation and how soon after irradiation it must be added to inhibit DNA fragmentation.

Figure 4 shows the results of experiments in which irradiated thymocytes were incubated with trolox for different lengths of time. At selected times (0.5–4 h) cells were removed from the medium containing trolox by centrifugation and incubated in fresh medium without trolox at 37°C in air containing 5% CO₂. DNA fragmentation was then determined 24 h after irradiation. As shown in Figure 4 the DNA fragmentation in thymocytes irradiated at 3 and 6 Gy was 68 and 76%, respectively. The extent of DNA fragmentation decreased as the incubation time with trolox increased, reaching a minimum after a 2-h incubation. DNA fragmentation in irradiated thymocytes after a 2-h incubation with trolox was 15%, the same level measured in unirradiated thymocytes treated the same way with trolox. These data indicate that a 2-h incubation with trolox is sufficient to block the DNA fragmentation measured at 24 h and that once the trolox-sensitive step is inhibited, fragmentation fails to occur even after trolox is removed.

To identify how early trolox must be present to block DNA fragmentation after irradiation, we added trolox to thymocytes during or at different

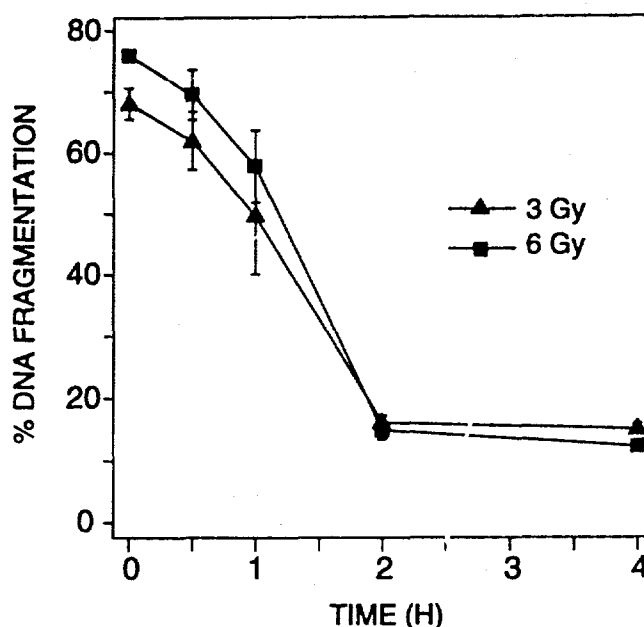


Figure 4. Inhibition of DNA fragmentation depends on the duration of trolox treatment. Irradiated thymocytes (3 and 6 Gy) were incubated in fresh medium with 10 mM trolox for different periods of time. At indicated times thymocytes were removed from the medium by centrifugation. The cells were resuspended in trolox-free medium and incubated under the conditions described in Figure 1. DNA fragmentation was determined 24 h after irradiation. The results are mean \pm SE from three independent experiments ($n=6$).

times following irradiation (6 Gy), and determined the extent of DNA fragmentation 8-h post-irradiation. The results shown in Figure 5 indicate that trolox does not inhibit DNA fragmentation when present only during irradiation. The addition of trolox within 30 min after irradiation was sufficient to inhibit almost completely the DNA fragmentation. Adding trolox 1 h after irradiation resulted in about 15% of the DNA becoming fragmented. Trolox added 2 or 4 h after irradiation resulted in 20 or 33% DNA fragmentation, respectively. Therefore, trolox completely inhibits DNA fragmentation only when added within 30 min after radiation exposure, suggesting that there is a critical event in the process of apoptosis within the first 30 min after irradiation that is sensitive to trolox.

We performed experiments to determine whether DNA fragmentation was correlated with changes in Ca²⁺ uptake into irradiated thymocytes. The amount of Ca²⁺ associated with irradiated thymocytes remained the same as unirradiated controls until 2 h post-irradiation (Figure 6). At 3 h the amount of Ca²⁺ associated with irradiated thymocytes was more than twice that of unirradiated

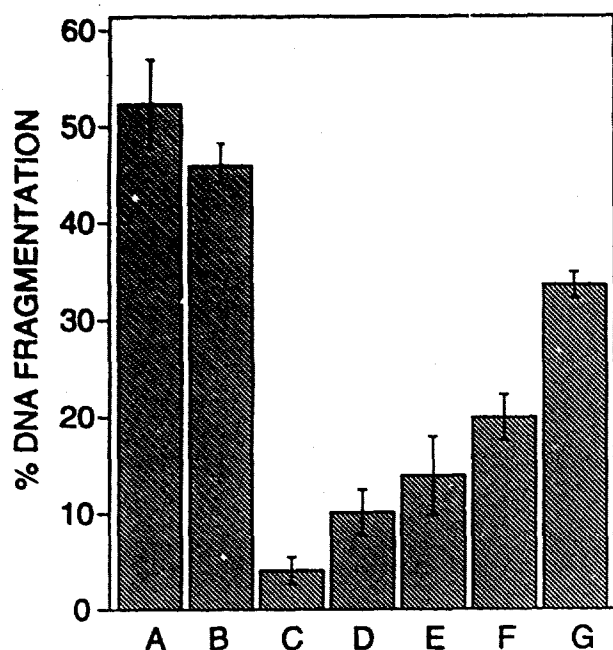


Figure 5. Inhibition of DNA fragmentation depends on timing of trolox treatment. Thymocytes were exposed to 6 Gy γ -radiation and incubated under the conditions described in Figure 1. In one set, thymocytes were irradiated in the presence of trolox. Immediately after irradiation the medium containing the trolox was removed by centrifugation and the incubation continued in trolox-free medium. The other sets were irradiated in medium without trolox; trolox (10 mM) was then added at the indicated times after irradiation and the incubation continued. DNA fragmentation was determined 8 h after irradiation. The results are mean \pm SE from three independent experiments ($n=6$). 6 Gy (bar A); trolox present during irradiation, then removed (bar B); trolox added to irradiated thymocytes at 0 min (bar C); 30 min (bar D); 1 h (bar E); 2 h (bar F); or 4 h (bar G) after irradiation.

thymocytes (261 ± 18 versus 101 ± 20 pmol/ 10^6 cells). The induction of DNA fragmentation and influx of Ca^{2+} appear concurrent (2–3 h post-irradiation; compare Figures 1a and 6). Interestingly, trolox completely inhibited the influx of Ca^{2+} in irradiated thymocytes.

We also studied the effect of trolox on DNA fragmentation following glucocorticoid treatment to determine whether the events blocked by trolox were specific for apoptosis stimulated by radiation. The results shown in Figure 7 indicate that trolox also inhibited DNA fragmentation induced in thymocytes by dexamethazone (compare lanes 4 and 5).

4. Discussion

Our findings demonstrate that trolox protects thymocytes from DNA fragmentation induced by γ -

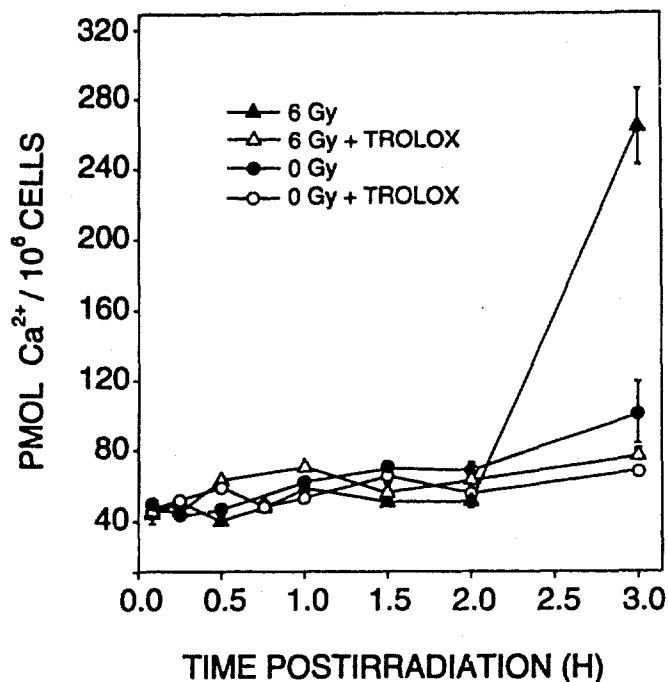


Figure 6. Trolox inhibits Ca^{2+} influx in irradiated thymocytes. Thymocytes (2×10^7 cells/ml) were incubated at 37°C in medium containing $^{45}\text{Ca}^{2+}$ ($10 \mu\text{Ci}/\text{ml}$) for 30 min before irradiation. Immediately after irradiation (6 Gy) trolox (10 mM) or buffer was added and the incubation continued. At selected times aliquots of the suspension were removed, the cells separated from the medium by centrifugation through oil, and radioactivity in the cell pellet determined by scintillation counting. Data represent the mean \pm SE of two independent experiments ($n=8$).

irradiation. DNA fragmentation was not inhibited if trolox was present only during irradiation (Figure 5), which suggests that the effectiveness of trolox in these experiments is not a result of its scavenging of free radicals generated during irradiation. On the other hand, trolox was very effective in inhibiting DNA fragmentation if added to the cells during the first 30 min after irradiation (Figure 5). The longer the addition of trolox was delayed, the less the level of protection observed, which suggests trolox inhibits an early step in a process that leads to an influx of Ca^{2+} and DNA fragmentation 2–3 h post-irradiation (Figures 1a and 6) (Ramakrishnan and Catravas 1992a, Story *et al.* 1992). Trolox need not be present continuously to exert its effect. Removing it from the irradiated cell suspension 2 h post-irradiation led to no greater degree of DNA fragmentation observed at 24 h than that measured in unirradiated controls (Figure 4). These results also suggest that the events responsible for DNA fragmentation begin soon after irradiation. Once these early steps are blocked the succeeding steps will not occur.

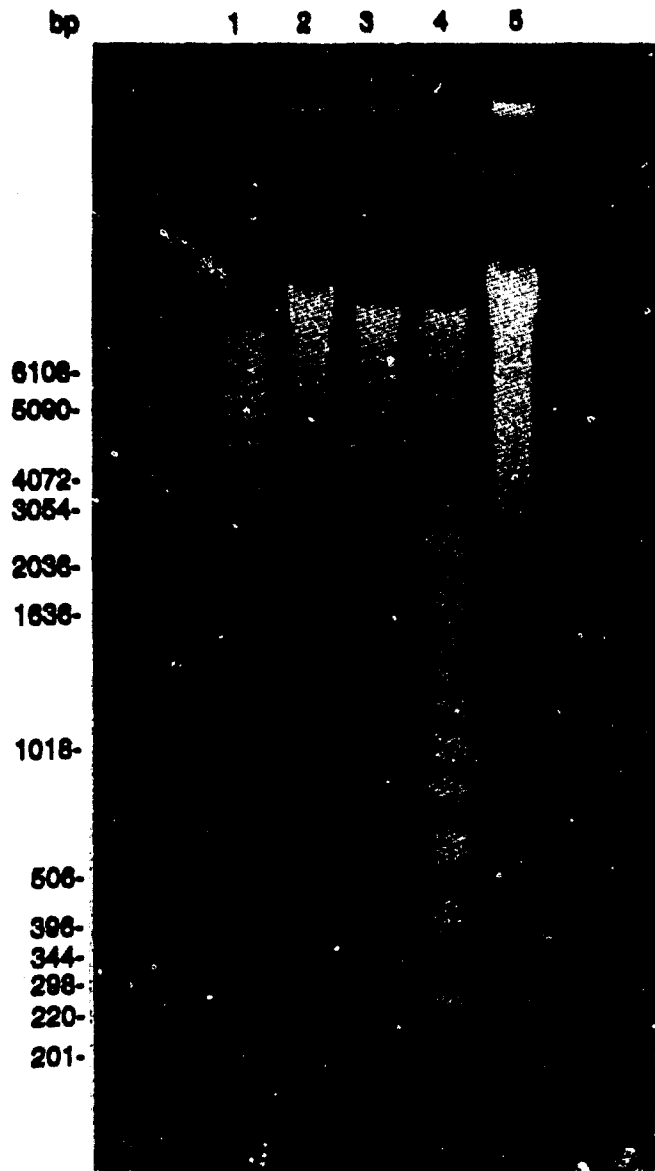


Figure 7. Trolox inhibits dexamethazone-induced DNA fragmentation. DNA was isolated from untreated and dexamethazone-treated (50 nM) thymocytes after 6 h incubation with or without 10 mM trolox and analysed by agarose gel electrophoresis. Lane 1, standard 1 kb DNA ladder; lane 2, control; lane 3, control + trolox; lane 4, 50 nM dexamethazone; and lane 5, dexamethazone + trolox.

Because trolox has been shown to be an inhibitor of membrane damage (Wu *et al.* 1990, Casini *et al.* 1985, Doha *et al.* 1985, Mickle *et al.* 1989, Barclay *et al.* 1984, Burton and Ingold 1985), it seems likely that trolox protects thymocytes by inhibiting structural and/or functional membrane changes responsible for triggering apoptosis. This interpretation is supported by our earlier studies with another lipophilic membrane-protecting antioxidant, dihydrolipoic acid, which also protects thymocytes from

radiation-induced DNA fragmentation (Ramakrishnan and Catravas 1992b). It is not surprising that events within membranes play a central role in radiation-induced apoptosis. Membrane damage has been reported in mammalian cells at radiation doses as low as 0.5 Gy (Parasassi *et al.* 1991). Human lymphocytes exhibit morphological changes in the plasma membrane within 15 min after irradiation with 0.5–1.5 Gy (Chandra and Stefani 1981, Stefani *et al.* 1977). Furthermore, changes in the structure and function of cellular membranes in irradiated thymocytes have been shown to occur much earlier than DNA fragmentation (Zherbin and Chukhlovin 1984, Yamada *et al.* 1969, Chandra and Stefani 1981, Sungurov and Sharlaeva 1988).

Our initial hypothesis of the mechanism of action of trolox was that it acted as an antioxidant to block lipid peroxidation and other membrane oxidations induced by radiation. However, our observation that trolox is also a potent inhibitor of glucocorticoid-induced DNA fragmentation (Figure 7) suggests that such a hypothesis might be too narrow to explain the trolox inhibition. Glucocorticoid-stimulated apoptosis is not known to involve changes in the membrane like those induced by radiation. However, little is known of the specific reactions that occur in the membrane during glucocorticoid stimulation. They may well involve changes in the oxidation state of reactants, which the antioxidant trolox could interfere with. The glucocorticoid experiments do not contradict the basic notion that trolox interferes with a membrane-related event early in the process of apoptosis. Apoptosis stimulated by glucocorticoids is a receptor-mediated process (Wyllie 1980). Glucocorticoids have been shown to impair membrane-related transport functions; cellular transport of amino acids, glucose, and nucleosides have been shown to be inhibited in thymocytes within 60 min after glucocorticoid treatment (Munck 1968, Makman *et al.* 1968, 1971).

Although it is not yet clear what membrane functions trolox protects to prevent the DNA degradation induced by radiation, our results strongly suggest trolox blocks some aspect of calcium metabolism associated with apoptosis. Many studies have shown that a critical event in apoptosis is an increase in cytosolic Ca^{2+} due to an internal mobilization of intracellular Ca^{2+} and an influx of extracellular Ca^{2+} (McConkey *et al.* 1989b, Story *et al.* 1992). Three hours post-irradiation, the amount of Ca^{2+} in irradiated thymocytes was more than twice that of those not irradiated (Figure 6). The fact that trolox blocked the influx of extracellular Ca^{2+} in irradiated thymocytes (Figure 6) suggests that trolox inhibits a change in membrane function that is

responsible for calcium mobilization during apoptosis. It may be that trolox protects calcium translocating mechanisms in the membrane that are otherwise altered by radiation. Indeed, it has been shown that vitamin E, from which trolox is derived, protects calcium translocases that are impaired during membrane damage (Ananieva *et al.* 1984).

In summary, this study supports the hypothesis that early events in apoptosis stimulated by physical agents or receptor-mediated processes occur at the membrane level. Trolox, an inhibitor of membrane damage, protects thymocytes from apoptosis. The critical lesion(s) blocked by trolox occur soon after stimulation. Once the lesion is prevented, trolox can be removed and apoptosis will not proceed. Experiments are currently in progress to identify the specific membrane events modulated by trolox to prevent apoptosis.

Acknowledgements

Trolox was kindly provided by Hoffman-La Roche. We thank W. Wolfe for his excellent technical assistance. The assistance of the Veterinary Sciences Department, Radiation Sources Department, and Information Services Department is also appreciated. This work was performed while Dr. Ramakrishnan held a National Research Council-AFRRRI Associateship, and the research was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency. Research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

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